



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 9/04, 15/53, C11D 3/386, A21D 2/26	A1	(11) International Publication Number: WO 95/29996 (43) International Publication Date: 9 November 1995 (09.11.95)
(21) International Application Number: PCT/DK95/00178 (22) International Filing Date: 3 May 1995 (03.05.95) (30) Priority Data: 504/94 3 May 1994 (03.05.94) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): OXENBØLL, Karen, M. [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). SI, Joan, Qi [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). AAGAARD, Jesper [DK/DK]; Novo Nordisk A/S, Novo Allé, DK-2880 Bagsværd (DK). (74) Common Representative: NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsværd (DK).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report.</i> <i>With amended claims and statement.</i>
(54) Title: ALKALINE GLUCOSE OXIDASE (57) Abstract A glucose oxidase characterized by a pH-optimum in the range pH 6-7, having more than 75 % of maximum activity at pH 8, determined at 30 °C with D-glucose as substrate.		

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ALKALINE GLUCOSE OXIDASE

FIELD OF INVENTION

The present invention relates to a novel glucose oxidase, to a process for its production and to its use in bleaching and detergent compositions, as well as to its use as a dough strengthener.

BACKGROUND OF THE INVENTION

Glucose oxidases are enzymes that catalyze the oxidation of glucose with oxygen whereby hydrogen peroxide is formed. Such enzymes are known from microbial, plant and animal origins, e.g., glucose oxidase from Aspergillus, Penicillium and Talaromyces. Glucose oxidase has been described as useful for various purposes, e.g., for bleaching purposes and in the baking industry, useful for strengthening the dough.

15 An example of a commercial glucose oxidase is Gluzyme™, an Aspergillus niger glucose oxidase, available from Novo Nordisk A/S. This and similar products from other commercial sources have an acidic pH optimum, typically around pH 5, which means that they are not very active in detergent solutions due to the alkaline character of the detergents.

20 It is an object of the present invention to provide novel glucose oxidases with improved performance in neutral and alkaline solutions.

SUMMARY OF THE INVENTION

25 In this invention it is surprisingly found that a glucose oxidase with a neutral pH optimum may be produced from strains of Cladosporium.

Accordingly, the present invention relates to a glucose oxidase characterized by a pH-optimum in the range pH 30 6-7, having more than 75% of maximum activity at pH 8, deter-

mined at 30°C with D-glucose as substrate.

BRIEF DESCRIPTION OF DRAWINGS

The present invention is further illustrated by reference to the accompanying drawings, in which

5 Fig. 1 shows the relation between pH and the glucose oxidase activity of a novel glucose oxidase according to the invention (the glucose oxidase obtained according to Example 1), with D-glucose as substrate in the presence of oxygen at 30°C, using a buffer system adjusted to predetermined pH values
10 of from pH 4 to pH 9.

Fig. 2 shows the dynamic shear storage modulus G' in gluten from 4 different doughs characterized with the following symbols:

□: a dough with no added conditioner (= reference)

15 ∇: a dough containing 20 ppm $KBrO_3$.

◇: a dough containing 500 UNITS per kg of flour of a glucose oxidase from A. niger.

△: a dough containing 500 UNITS per kg of flour of a glucose oxidase of the invention, obtained as described in Example 1.

20 Fig. 3 shows the viscoelastic phase angle δ in gluten from 4 different doughs characterized with the following symbols:

□: a dough with no added conditioner (= reference)

∇: a dough containing 20 ppm $KBrO_3$.

25 ◇: a dough containing 500 UNITS per kg of flour of a glucose oxidase from A. niger.

△: a dough containing 500 UNITS per kg of flour of a glucose oxidase of the invention, obtained as described in Example 1.

DETAILED DESCRIPTION OF THE INVENTION

30 Microorganism

According to the invention, glucose oxidase is obtainable from a glucose oxidase producing strain of Clado-

sporium, preferably Cladosporium oxysporum.

The genus Cladosporium is characterized primarily by the formation of conidia in branching chains, which are very fragile and readily break up into units. The conidia can be either hyaline or pigmented, smooth or roughened, continuous or septate. The conidiophores are erect and pigmented, branching irregularly at the apex. Cladosporium oxysporum is further described by M. B. Ellis in Dermatiaceous Hyphomycetes, 1971, p. 312, CAB International, UK.

10 A strain representative of Cladosporium oxysporum has been deposited according to the Budapest Treaty on the International Recognition of the Deposits of Microorganisms for the Purpose of Patent Procedures, on 25 March 1994, at Centraal-bureau voor Schimmelcultures (CBS), under Accession No. CBS
15 163.94.

Production of Glucose Oxidase

Glucose oxidase of the invention may be produced by aerobic cultivation of the above mentioned microbial strain on a nutrient medium containing suitable carbon and nitrogen
20 sources, such media being known in the art. A temperature in the range of from 20°C to 30°C is suitable for growth and glucose oxidase production.

Alternatively, glucose oxidase of the invention can be produced by aerobic cultivation of a transformed host
25 organism containing the appropriate genetic information from the above mentioned strain. Such transformants can be prepared and cultivated by methods known in the art:

Cloning a DNA Sequence Encoding a Glucose Oxidase

The DNA sequence encoding a glucose oxidase of the
30 invention may be isolated from any cell or microorganism producing the glucose oxidase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the glucose oxidase to

be studied. Then, if the amino acid sequence of the glucose oxidase is known, homologous, labelled oligonucleotide probes may be synthesized and used to identify glucose oxidase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labelled oligonucleotide probe containing sequences homologous to a known glucose oxidase gene could be used as a probe to identify glucose oxidase-encoding clones, using hybridization and washing conditions of lower stringency. According to the present invention preferred probes may be constructed on the basis of SEQ ID No. 1.

Yet another method for identifying glucose oxidase-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming glucose oxidase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for glucose oxidase thereby allowing clones expressing the glucose oxidase to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by S.L. Beaucage and M.H. Caruthers in Tetrahedron Letters 22, 1981, pp. 1859-1869 or the method described by Matthes et al. in The EMBO J. 3, 1984, pp. 801-805. In the phosphoramidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. in Science 239, 1988, pp. 487-

491.

Expression of Glucose Oxidase

According to the invention, a glucose oxidase-encoding DNA sequence produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

10 The recombinant expression vector carrying the DNA sequence encoding a glucose oxidase of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector
15 may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one
20 which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be
25 any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding a glucose oxidase of the invention, especially in a bacterial host, are the promoter of the
30 lac operon of E.coli, the Streptomyces coelicolor agarase gene dagA promoters, the promoters of the Bacillus licheniformis α -amylase gene (amyL), the promoters of the Bacillus stearothermophilus maltogenic amylase gene (amyM), the promoters of the
35 Bacillus Amylolyquefaciens α -amylase (amyQ), the promoters of

the Bacillus subtilis xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral α -amylase, 5 A. niger acid stable α -amylase, A. niger glucoamylase, Rhizomucor miehei lipase, A. oryzae alkaline protease, A. oryzae triose phosphate isomerase or A. nidulans acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in 10 eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the glucose oxidase of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence 15 enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g., a gene the product of which complements a defect in the 20 host cell, such as the dal genes from B. subtilis or B. licheniformis, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise Aspergillus selection markers such as amdS, argB, niaD and sC, a marker 25 giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, e.g., as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g., when using certain bacteria as host 30 cells, it is generally preferred that the expression is extracellular.

Procedures suitable for constructing vectors of the invention encoding a glucose oxidase and containing the promoter, terminator and other elements, respectively, are well 35 known to persons skilled in the art (cf., for instance, Sambrook et al. in Molecular Cloning: A Laboratory Manual, 2nd

Ed., Cold Spring Harbor, 1989).

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a glucose oxidase of the invention. The cell may be transformed with the DNA construct of the invention encoding the glucose oxidase conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g., by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but is preferably a microbial cell, e.g., a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are grampositive bacteria such as Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans, Bacillus lautus, Bacillus megaterium, Bacillus thuringiensis, or Streptomyces lividans or Streptomyces murinus, or gramnegative bacteria such as E.coli.

The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known per se.

The yeast organism may favourably be selected from a species of Saccharomyces or Schizosaccharomyces, e.g., Saccharomyces cerevisiae. The filamentous fungus may advantageously belong to a species of Aspergillus, e.g., Aspergillus oryzae or Aspergillus niger. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a

manner known per se. A suitable procedure for transformation of Aspergillus host cells is described in EP 238 023.

In a yet further aspect, the present invention relates to a method of producing a glucose oxidase of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the glucose oxidase and recovering the glucose oxidase from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the glucose oxidase of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g., as described in catalogues of the American Type Culture Collection).

The glucose oxidase secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

Assay for Glucose Oxidase Activity

Glucose oxidase activity is determined in the following way: Glucose oxidase oxidizes D-glucose in the presence of oxygen producing hydrogen peroxide. The hydrogen peroxide formed, in the presence of peroxidase, oxidizes ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate)). The greenish-blue colour resulting after a fixed reaction time measured as the absorbance at 418 nm is a function of the amount of hydrogen peroxide. In the assay the following concentrations, pH, temperature and reaction time are used: D-glucose: 100 mM; ABTS: 0.4 mM; phosphate buffer: 100 mM; pH: 7.0; temperature: 30°C; reaction time: 20 min. The activity of

glucose oxidase is given in UNITS (1 UNIT is the amount of glucose oxidase which under the above standard conditions forms 1 μ mole of hydrogen peroxide per minute).

Physico-chemical Properties of the Glucose Oxidase

5 The glucose oxidase of the invention (obtained according to Example 1) possesses the following property:

A pH profile as shown in Fig. 1, which was determined at 30°C in the pH range of from pH 4 to pH 9. The assay for glucose oxidase activity described above was run in a buffer of 10 CH_3COOH , KH_2PO_4 and H_3BO_3 at a glucose level of 100 mM. The reaction time was 20 minutes. The peroxidase reaction was run separately after the incubation of the glucose oxidase. It appears from Fig. 1 that the enzyme possesses glucose oxidase activity from approximately pH 4 to above pH 9, having optimum 15 in the range pH 6-7, and more than 75% of maximum activity at pH 8.

N-terminal.

An amino acid sequence of an enzyme may be determined using standard methods for obtaining and sequencing peptides, 20 for example as described by Findlay & Geisow (Eds.), Protein Sequencing - a Practical Approach, 1989, IRL Press.

The N-terminal amino acid sequence of the glucose oxidase obtained from Cladosporium oxysporum, CBS 163.94, fermented and purified as described in Example 1, was found to 25 be :

Ala-Ser-Pro-Ala-Glu-Pro-Pro-Val-Val-Ala-Ala (SEQ ID No. 1).

A peptide is considered to be X% homologous to the parent glucose oxidase if a comparison of the respective amino acid sequences, performed via known algorithms, such as the one 30 described by Lipman and Pearson in Science 227, 1985, p. 1435, reveals an identity of X%.

The present invention relates to a glucose oxidase comprising an N-terminal amino acid sequence identical to that shown in SEQ ID No. 1 or a glucose oxidase being at least 70%

homologous with SEQ ID No.1 in the N-terminal, preferably being at least 80% homologous with SEQ ID No.1 in the N-terminal, more preferably being at least 90% homologous with SEQ ID No. 1.

5 Immunochemical Properties

The immunochemical properties can be determined immunologically by cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to I. M. Roitt; Immunology, Gower Medical Publishing (1985) and N. H. Axelsen; Handbook of Immunoprecipitation-in-Gel Techniques; Blackwell Scientific Publications (1983), Chapters 5 and 14. The terms "antigenic identity" and "partial antigenic identity" are described in the same book, 15 Chapters 5, 19 and 20.

Monospecific antiserum may be generated according to the above mentioned method by immunizing rabbits with a glucose oxidase of the invention. The immunogen is mixed with Freund's adjuvant and injected subcutaneously into rabbits every second 20 week. Antiserum is obtained after a total immunization period of 8 weeks, and immunoglobulin prepared therefrom as described by N. H. Axelsen, supra.

According to the invention a glucose oxidase displaying immunochemical cross-reactivity with an antibody 25 raised against a glucose oxidase obtainable from a strain of the genus Cladosporium is preferred, in particular a glucose oxidase having immunochemical properties identical to those of a glucose oxidase obtainable from a strain of the genus Cladosporium.

30 Industrial Applications

The glucose oxidase of the invention possesses valuable properties allowing for various industrial applications. In particular the enzyme, in having activity in the alkaline region, finds potential application in washing

detergent compositions as a hydrogen peroxide source, used alone or preferably together with a peroxidase, more preferably used together with a peroxidase and an oxidizable substrate such as an organic compound, such as a phenolic compound, e.g., p-hydroxybenzenesulfonate, or one of the compounds disclosed in WO 94/12621.

The enzyme may also be very useful in the baking industry due to its excellent ability for improving the properties of doughs/breads (for documentation see Example 2 + 3).

The enzyme also has many potential applications in the personal care area, for example in personal care products such as tooth paste, mouthwash, denture cleaner, liquid soap, skin care creams and lotions, hair care and body care formulations, and solutions for cleaning contact lenses. In particular the glucose oxidase of the invention may be very useful in tooth paste, alone or together with other enzymes, preferably together with an amyloglucosidase and a lactoperoxidase as such a combination of enzymes forms a very efficient antibacterial system:

Polysaccharides from plaques --> (Amyloglucosidase)

Glucose --> (Glucose oxidase of the invention)

Gluconic acid + H_2O_2 ;

the formed hydrogen peroxide may react with thiocyanate in the following way:

H_2O_2 + SCN^- --> (Lactoperoxidase)

$OSCN^-$, in which $OSCN^-$ is a bacteriostatic agent.

Gluten Strengthening

It is generally known that dough stability is one of the most important characteristics of a baking dough. Stable dough is important for both large scale and small scale applications. A strong dough will exhibit a greater tolerance of mixing time, of proofing time and of mechanical vibrations during dough transport, so that the baked product maintains its

good quality. A weak dough will possess less tolerance. Therefore, a strong dough is generally preferred in most bread-making. A high gluten content and a good gluten quality form a stronger dough than a dough made from a low protein content or
5 with poor gluten quality. In other words, a strong gluten network results in a strong dough, which has superior rheological and handling properties.

Dough "conditioners" to strengthen the gluten have long been used. The non-specific oxidants, such as bromate,
10 ascorbic acid and peroxides have the gluten strengthening effect. It has been suggested that these conditioners induce the interprotein bonds which strengthen the gluten, thereby the dough. Enzymes used as dough conditioners are also known, e.g. glucose oxidase from Aspergillus niger.

15 The strengthening effect of a given dough conditioner on wheat flour dough or gluten dough may be measured by dynamic rheological measurements. These measurements are able to show the strength of a dough, under oscillation. Both wheat flour dough and gluten dough are viscoelastic materials. In oscillatory measurements, the viscoelastic properties of a wheat
20 dough and a gluten dough can be divided into two components, the dynamic shear storage modulus G' and the dynamic shear loss modulus G'' . The ratio of the loss and the storage moduli is numerically equal to the tangent of the viscoelastic phase angle δ . An increase in the storage modulus G' and a decrease
25 in the phase angle δ indicate a stronger and more elastic dough.

Baking industry/Additional Enzyme Activities

While the bread-improving composition or additive may
30 comprise a glucose oxidase as the only enzyme added, the properties of dough and/or baked products may be further improved when the glucose oxidase is used in combination with one or more additional enzymes.

The additional enzyme(s) may either be one or more
35 enzymes present in the glucose oxidase preparation recovered

from the organism producing it, or may, more preferably, be added to the bread-improving composition or additive.

In a preferred embodiment, the additional enzyme is selected from the group consisting of an amylase, in particular an amyloglucosidase, an α -amylase or a maltogenic exo-amylase (at present contemplated useful for providing sugars fermentable by yeast), a peptidase, a maltogenase, a lipase, a cellulase, a hemicellulase, in particular a xylanase, a protease (at present contemplated useful for gluten weakening in particular when using hard wheat flour), and an oxidoreductase, e.g., a peroxidase, a laccase, a protein disulfide isomerase, e.g., a protein disulfide isomerase as disclosed in WO 95/00636, or an additional glucose oxidase.

In a more preferred embodiment, the additional enzyme is selected from the group consisting of a hemicellulase, in particular a xylanase, and an amylase, e.g., an amyloglucosidase, an α -amylase or a maltogenic exo-amylase.

The additional enzyme is preferably of microbial (bacterial, yeast or fungal) origin and may be obtained by techniques conventionally used in the art.

The amylase may be derived from a bacterium or a fungus, in particular from a strain of Aspergillus, preferably a strain of Aspergillus niger or Aspergillus oryzae, or from a strain of Bacillus. Commercially available α -amylases useful for the present purpose are Fungamyl™ (an Aspergillus oryzae α -amylase, available from Novo Nordisk A/S, Denmark), Novamyl™ (a Bacillus stearothermophilus maltogenic exo-amylase, available from Novo Nordisk A/S, Denmark), and BAN™ (a Bacillus amyloliquefaciens α -amylase, available from Novo Nordisk A/S, Denmark). The amyloglucosidase may in particular be AMG™ (an A. niger amyloglucosidase, available from Novo Nordisk A/S, Denmark). Other useful amylase products include Grindamyl™ A 1000 or A 5000 (available from Grindsted Products, Denmark) and Amylase™ H or Amylase™ P (available from Gist-Brocades, The Netherlands).

The additional glucose oxidase may be a fungal

glucose oxidase, in particular Gluzyne™ (an Aspergillus niger glucose oxidase, available from Novo Nordisk A/S, Denmark).

The protease may in particular be Neutrase™ (available from Novo Nordisk A/S, Denmark).

5 The lipase may be derived from a strain of Thermomyces, a strain of Rhizomucor, a strain of Candida, a strain of Aspergillus, a strain of Rhizopus, or a strain of Pseudomonas. In particular the lipase may be derived from a strain of Thermomyces lanuginosus, a strain of Rhizomucor miehei, a
10 strain of Candida antarctica, a strain of Aspergillus niger, or a strain of Pseudomonas cepacia. In specific embodiments, the lipase may be Lipase A or Lipase B derived from a strain of Candida antarctica as described in WO 88/02775, or the lipase may be derived from a strain of Rhizomucor miehei as described
15 in EP 238,023, or a strain of Humicola lanuginosa described in EP 305,216, or a strain of Pseudomonas cepacia as described in EP 214,761 and WO 89/01032.

Besides the above mentioned additional enzymes, a microbial produced glucose oxidase preparation may contain
20 varying minor amounts of other enzymatic activities inherently produced by the producer organism in question.

Detergent Compositions

According to the invention, the glucose oxidase may typically be a component (a hydrogen peroxide source) of a
25 detergent composition, e.g., a laundry detergent composition or a dishwashing detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in
30 US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50
35 ethylene oxide units; ethoxylated fatty alcohols in which the

alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or amphoteric (zwitterionic). The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzene-sulfonate (LAS), alpha-olefinsulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkanesulfonates (SAS), alpha-sulfo fatty acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), alcohol propoxylate, carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as pullulanase, esterase, lipase, cutinase, protease, cellulase, or peroxidase.

Normally the detergent contains 1-65% of a detergent builder, but some dishwashing detergents may contain even up to 90% of a detergent builder, or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate,

nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

5 The detergent builders may be subdivided into phosphorus-containing and non-phosphorous-containing types. Examples of phosphorus-containing inorganic alkaline detergent builders include the water-soluble salts, especially alkali metal pyrophosphates, orthophosphates, polyphosphates and
10 phosphonates. Examples of non-phosphorus-containing inorganic builders include water-soluble alkali metal carbonates, borates and silicates as well as layered disilicates and the various types of water-insoluble crystalline or amorphous aluminosilicates of which zeolites is the best known representative.

15 Examples of suitable organic builders include alkali metal, ammonium or substituted ammonium salts of succinates, malonates, fatty acid malonates, fatty acid sulphonates, carboxymethoxy succinates, polyacetates, carboxylates, polycarboxylates, aminopolycarboxylates and polyacetyl carboxylates.

20 The detergent may also be unbuilt, i.e. essentially free of detergent builder.

 The detergent may comprise one or more polymers. Examples are carboxymethylcellulose (CMC), poly(vinylpyrrolidone) (PVP), polyethyleneglycol (PEG), poly(vinyl
25 alcohol) (PVA), polycarboxylates such as polyacrylates, polymaleates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

 The detergent composition may additionally contain other bleaching agents of the chlorine/bromine-type or the
30 oxygen-type. The bleaching agents may be coated or encapsulated. Examples of inorganic chlorine/bromine-type bleaches are lithium, sodium or calcium hypochlorite or hypobromite as well as chlorinated trisodium phosphate.

 Examples of organic chlorine/bromine-type bleaches
35 are heterocyclic N-bromo and N-chloro imides such as trichloroisocyanuric, tribromoisocyanuric, dibromoisocyanuric and

dichloroisocyanuric acids, and salts thereof with water solubilizing cations such as potassium and sodium. Hydantoin compounds are also suitable. The bleaching system may also comprise peroxyacids of, e.g., the amide, imide, or sulfone type.

In dishwashing detergents the oxygen bleaches are preferred, for example in the form of an inorganic persalt, preferably with a bleach precursor or as a peroxy acid compound. Typical examples of suitable peroxy bleach compounds are
10 alkali metal perborates, both tetrahydrates and monohydrates, alkali metal percarbonates, persilicates and perphosphates. Preferred activator materials are TAED or NOBS.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing
15 agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708. The enzymes of the invention may also
20 be stabilized by adding reversible enzyme inhibitors, e.g., of the protein type as described in EP 0 544 777 B1.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, deflocculant material, foam boosters/foam
25 depressors (in dishwashing detergents foam depressors), suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, dehydrating agents, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the
30 range of 7-11.

Particular forms of laundry detergent compositions within the scope of the invention include:

- 1) A detergent composition formulated as a granulate having a

bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	7	-	12%
5	Alcohol ethoxysulfate (e.g. C ₁₂₋₁₈ alcohol, 1-2 EO) or alkyl sulfate (e.g. C ₁₆₋₁₈)	1	-	4%
	Alcohol ethoxylate (e.g. C ₁₄₋₁₅ alcohol, 7 EO)	5	-	9%
	Sodium carbonate (as Na ₂ CO ₃)	14	-	20%
10	Soluble silicate (as Na ₂ O, 2SiO ₂)	2	-	6%
	Zeolite (as NaAlSiO ₄)	15	-	22%
	Sodium sulfate (as Na ₂ SO ₄)	0	-	6%
	Sodium citrate/citric acid (as C ₆ H ₅ Na ₃ O ₇ /C ₆ H ₈ O ₇)	0	-	15%
15	Sodium perborate (as NaBO ₃ ·H ₂ O)	11	-	18%
	TAED	2	-	6%
	Carboxymethylcellulose	0	-	2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0	-	3%
20	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
	Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0	-	5%

25 2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	6	-	11%
30	Alcohol ethoxysulfate (e.g. C ₁₂₋₁₈ alcohol, 1-2 EO or alkyl sulfate (e.g. C ₁₆₋₁₈))	1	-	3%
	Alcohol ethoxylate (e.g. C ₁₄₋₁₅ alcohol, 7 EO)	5	-	9%
35	Sodium carbonate (as Na ₂ CO ₃)	15	-	21%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	1	-	4%
	Zeolite (as NaAlSiO ₄)	24	-	34%

	Sodium sulfate (as Na_2SO_4)	4 - 10%
	Sodium citrate/citric acid (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7/\text{C}_6\text{H}_8\text{O}_7$)	0 - 15%
	Carboxymethylcellulose	0 - 2%
5	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
10	Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	5 - 9%
15	Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	7 - 14%
	Soap as fatty acid (e.g. C_{16-22} fatty acid)	1 - 3%
	Sodium carbonate (as Na_2CO_3)	10 - 17%
20	Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$)	3 - 9%
	Zeolite (as NaAlSiO_4)	23 - 33%
	Sodium sulfate (as Na_2SO_4)	0 - 4%
	Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$)	8 - 16%
	TAED	2 - 8%
25	Phosphonate (e.g. EDTMPA)	0 - 1%
	Carboxymethylcellulose	0 - 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
30	Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
	Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	Linear alkylbenzenesulfonate (calculated as acid)	8	- 12%
5	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO)	10	- 25%
	Sodium carbonate (as Na ₂ CO ₃)	14	- 22%
	Soluble silicate (as Na ₂ O, 2SiO ₂)	1	- 5%
	Zeolite (as NaAlSiO ₄)	25	- 35%
10	Sodium sulfate (as Na ₂ SO ₄)	0	- 10%
	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1	- 3%
15	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

5) An aqueous liquid detergent composition comprising

20	Linear alkylbenzenesulfonate (calculated as acid)	15	- 21%
	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO or C ₁₂₋₁₅ alcohol, 5 EO)	12	- 18%
	Soap as fatty acid (e.g. oleic acid)	3	- 13%
25	Alkenylsuccinic acid (C ₁₂₋₁₄)	0	- 13%
	Aminoethanol	8	- 18%
	Citric acid	2	- 8%
	Phosphonate	0	- 3%
	Polymers (e.g. PVP, PEG)	0	- 3%
30	Borate (as B ₄ O ₇)	0	- 2%
	Ethanol	0	- 3%
	Propylene glycol	8	- 14%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%

Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0	-	5%
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6) An aqueous structured liquid detergent composition comprising

Linear alkylbenzenesulfonate (calculated as acid)	15	-	21%
Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO, or C ₁₂₋₁₅ alcohol, 5 EO)	3	-	9%
Soap as fatty acid (e.g. oleic acid)	3	-	10%
Zeolite (as NaAlSiO ₄)	14	-	22%
Potassium citrate	9	-	18%
Borate (as B ₄ O ₇)	0	-	2%
Carboxymethylcellulose	0	-	2%
Polymers (e.g. PEG, PVP)	0	-	3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0	-	3%
Glycerol	0	-	5%
Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0	-	5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Fatty alcohol sulfate	5	-	10%
Ethoxylated fatty acid monoethanolamide	3	-	9%
Soap as fatty acid	0	-	3%
Sodium carbonate (as Na ₂ CO ₃)	5	-	10%
Soluble silicate (as Na ₂ O, 2SiO ₂)	1	-	4%

	Zeolite (as NaAlSiO_4)	20	- 40%
	Sodium sulfate (as Na_2SO_4)	2	- 8%
	Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$)	12	- 18%
	TAED	2	- 7%
5	Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
10	Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0	- 5%

8) A detergent composition formulated as a granulate comprising

	Linear alkylbenzenesulfonate (calculated as acid)	8	- 14%
15	Ethoxylated fatty acid monoethanol-amide	5	- 11%
	Soap as fatty acid	0	- 3%
	Sodium carbonate (as Na_2CO_3)	4	- 10%
	Soluble silicate (as $\text{Na}_2\text{O} \cdot 2\text{SiO}_2$)	1	- 4%
20	Zeolite (as NaAlSiO_4)	30	- 50%
	Sodium sulfate (as Na_2SO_4)	3	- 11%
	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$)	5	- 12%
	Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1	- 5%
25	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. suds suppressors, perfume)	0	- 5%

9) A detergent composition formulated as a granulate comprising

30	Linear alkylbenzenesulfonate (calculated as acid)	6	- 12%
	Nonionic surfactant	1	- 4%
	Soap as fatty acid	2	- 6%
	Sodium carbonate (as Na_2CO_3)	14	- 22%

	Zeolite (as NaAlSiO_4)	18	- 32%
	Sodium sulfate (as Na_2SO_4)	5	- 20%
	Sodium citrate (as $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$)	3	- 8%
	Sodium perborate (as $\text{NaBO}_3 \cdot \text{H}_2\text{O}$)	4	- 9%
5	Bleach activator (e.g. NOBS or TAED)	1	- 5%
	Carboxymethylcellulose	0	- 2%
	Polymers (e.g. polycarboxylate or PEG)	1	- 5%
10	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
	Minor ingredients (e.g. optical brightener, perfume)	0	- 5%

10) An aqueous liquid detergent composition comprising

15	Linear alkylbenzenesulfonate (calculated as acid)	15	- 23%
	Alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8	- 15%
20	Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3	- 9%
	Soap as fatty acid (e.g. lauric acid)	0	- 3%
	Aminoethanol	1	- 5%
25	Sodium citrate	5	- 10%
	Hydrotrope (e.g. sodium toluenesulfonate)	2	- 6%
	Borate (as B_4O_7)	0	- 2%
	Carboxymethylcellulose	0	- 1%
30	Ethanol	1	- 3%
	Propylene glycol	2	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
35	Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0	- 5%

11) An aqueous liquid detergent composition comprising

	Linear alkylbenzenesulfonate (calculated as acid)	20	- 32%
5	Alcohol ethoxylate (e.g. C ₁₂₋₁₅ alcohol, 7 EO, or C ₁₂₋₁₅ alcohol, 5 EO)	6	- 12%
	Aminoethanol	2	- 6%
	Citric acid	8	- 14%
	Borate (as B ₄ O ₇)	1	- 3%
10	Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0	- 3%
15	Glycerol	3	- 8%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%
20	Minor ingredients (e.g. hydro-tropes, dispersants, perfume, optical brighteners)	0	- 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

25	Anionic surfactant (linear alkylbenzenesulfonate, alkyl sulfate, alpha-olefinsulfonate, alpha-sulfo fatty acid methyl esters, alkanesulfonates, soap)	25	- 40%
	Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 10%
30	Sodium carbonate (as Na ₂ CO ₃)	8	- 25%
	Soluble silicates (as Na ₂ O, 2SiO ₂)	5	- 15%
	Sodium sulfate (as Na ₂ SO ₄)	0	- 5%
	Zeolite (as NaAlSiO ₄)	15	- 28%
	Sodium perborate (as NaBO ₃ ·4H ₂ O)	0	- 20%
35	Bleach activator (TAED or NOBS)	0	- 5%
	Enzymes (calculated as pure enzyme protein)	0.0001	- 0.1%

Minor ingredients (e.g. perfume, optical brighteners)	0	-	3%
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13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzenesulfonate is replaced by (C₁₂-C₁₈) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	(C ₁₂ -C ₁₈) alkyl sulfate	9	-	15%
	Alcohol ethoxylate	3	-	6%
10	Polyhydroxy alkyl fatty acid amide	1	-	5%
	Zeolite (as NaAlSiO ₄)	10	-	20%
	Layered disilicate (e.g. SK56 from Hoechst)	10	-	20%
	Sodium carbonate (as Na ₂ CO ₃)	3	-	12%
15	Soluble silicate (as Na ₂ O, 2SiO ₂)	0	-	6%
	Sodium citrate	4	-	8%
	Sodium percarbonate	13	-	22%
	TAED	3	-	8%
20	Polymers (e.g. polycarboxylates and PVP)	0	-	5%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
25	Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0	-	5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

	(C ₁₂ -C ₁₈) alkyl sulfate	4	-	8%
	Alcohol ethoxylate	11	-	15%
30	Soap	1	-	4%
	Zeolite MAP or zeolite A	35	-	45%

	Sodium carbonate (as Na_2CO_3)	2	-	8%
	Soluble silicate (as $\text{Na}_2\text{O}, 2\text{SiO}_2$)	0	-	4%
	Sodium percarbonate	13	-	22%
	TAED	1	-	8%
5	Carboxymethyl cellulose	0	-	3%
	Polymers (e.g. polycarboxylates and PVP)	0	-	3%
	Enzymes (calculated as pure enzyme protein)	0.0001	-	0.1%
10	Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0	-	3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified
15 bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst.
20 The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g.,
25 linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

Particular forms of dishwashing detergent compositions within the scope of the invention include:

1) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	0.4	- 2.5%
	Sodium metasilicate	0	- 20%
	Sodium disilicate	3	- 20%
5	Sodium triphosphate	20	- 40%
	Sodium carbonate	0	- 20%
	Sodium perborate	2	- 9%
	Tetraacetythylenediamine (TAED)	1	- 4%
	Sodium sulphate	5	- 33%
10	Enzymes	0.0001	- 0.1%

2) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant (e.g. alcohol ethoxylate)	1	- 2%
	Sodium disilicate	2	- 30%
15	Sodium carbonate	10	- 50%
	Sodium phosphonate	0	- 5%
	Trisodium citrate dihydrate	9	- 30%
	Nitrilotrisodium acetate (NTA)	0	- 20%
	Sodium perborate monohydrate	5	- 10%
20	Tetraacetythylenediamine (TAED)	1	- 2%
	Polyacrylate polymer (e.g. maleic acid/acrylic acid co-polymer)	6	- 25%
	Enzymes	0.0001	- 0.1%
25	Perfume	0.1	- 0.5%
	Water	5	- 10

3) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	0.5	- 2.0%
	Sodium disilicate	25	- 40%
30	Sodium citrate	30	- 55%

	Sodium carbonate	0	- 29%
	Sodium bicarbonate	0	- 20%
	Sodium perborate monohydrate	0	- 15%
	Tetraacetylenethylenediamine (TAED)	0	- 6%
5	Maleic acid/acrylic acid copolymer	0	- 5%
	Clay	1	- 3%
	Poly(amino acids)	0	- 20%
	Sodium polyacrylate	0	- 8%
10	Enzymes	0.0001	- 0.1%

4) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	1	- 2%
	Zeolite MAP	15	- 42%
	Sodium disilicate	30	- 34%
15	Sodium citrate	0	- 12%
	Sodium carbonate	0	- 20%
	Sodium perborate monohydrate	7	- 15%
	Tetraacetylenethylenediamine (TAED)	0	- 3%
	Polymer	0	- 4%
20	Maleic acid/acrylic acid copolymer	0	- 5%
	Organic phosphonate	0	- 4%
	Clay	1	- 2%
	Enzymes	0.0001	- 0.1%
	Sodium sulphate	Balance	

25 5) POWDER AUTOMATIC DISHWASHING COMPOSITION

	Nonionic surfactant	1	- 7%
	Sodium disilicate	18	- 30%
	Trisodium citrate	10	- 24%
	Sodium carbonate	12	- 20%
30	Monopersulphate ($2 \text{ KHSO}_5 \cdot \text{KHSO}_4 \cdot \text{K}_2\text{SO}_4$)	15	- 21%

	Bleach stabilizer	0.1	-	2%
	Maleic acid/acrylic acid copolymer	0	-	6%
	Diethylenetriaminepentaacetate, pentasodium salt	0	-	2.5%
5	Enzymes	0.0001	-	0.1%
	Sodium sulphate, water	Balance		

6) POWDER AND LIQUID DISHWASHING COMPOSITION WITH CLEANING SURFACTANT SYSTEM

	Nonionic surfactant	0	-	1.5%
10	Octadecyl dimethylamine N-oxide dihydrate	0	-	5%
15	80:20 wt.C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dihydrate	0	-	4%
20	70:30 wt.C18/C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous	0	-	5%
	C ₁₃ -C ₁₅ alkyl ethoxysulfate with an average degree of ethoxylation of 3	0	-	10%
	C ₁₂ -C ₁₅ alkyl ethoxysulfate with an average degree of ethoxylation of 3	0	-	5%
25	C ₁₃ -C ₁₅ ethoxylated alcohol with an average degree of ethoxylation of 12	0	-	5%
30	A blend of C ₁₂ -C ₁₅ ethoxylated alcohols with an average degree of ethoxylation of 9	0	-	6.5%
	A blend of C ₁₃ -C ₁₅ ethoxylated alcohols with an average degree of ethoxylation of 30	0	-	4%
	Sodium disilicate	0	-	33%
35	Sodium tripolyphosphate	0	-	46%
	Sodium citrate	0	-	29%
	Citric acid	0	-	29%
	Sodium carbonate	0	-	20%

	Sodium perborate monohydrate	0	- 11.5%
	Tetraacetylenehydrazine (TAED)	0	- 4%
	Maleic acid/acrylic acid copolymer	0	- 7.5%
	Sodium sulphate	0	- 12.5%
5	Enzymes	0.0001	- 0.1%

7) NON-AQUEOUS LIQUID AUTOMATIC DISHWASHING COMPOSITION

	Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0	- 10.0%
	Alkali metal silicate	3.0	- 15.0%
10	Alkali metal phosphate	20.0	- 40.0%
	Liquid carrier selected from higher glycols, polyglycols, polyoxides, glycolethers	25.0	- 45.0%
15	Stabilizer (e.g. a partial ester of phosphoric acid and a C ₁₆ -C ₁₈ alkanol)	0.5	- 7.0%
	Foam suppressor (e.g. silicone)	0	- 1.5%
	Enzymes	0.0001	- 0.1%

8) NON-AQUEOUS LIQUID DISHWASHING COMPOSITION

20	Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0	- 10.0%
	Sodium silicate	3.0	- 15.0%
	Alkali metal carbonate	7.0	- 20.0%
	Sodium citrate	0.0	- 1.5%
25	Stabilizing system (e.g. mixtures of finely divided silicone and low molecular weight dialkyl polyglycol ethers)	0.5	- 7.0%
30	Low molecule weight polyacrylate polymer	5.0	- 15.0%
	Clay gel thickener (e.g. bentonite)	0.0	- 10.0%
	Hydroxypropyl cellulose polymer	0.0	- 0.6%
	Enzymes	0.0001	- 0.1%

Liquid carrier selected from higher lycols, polyglycols, polyoxides and glycol ethers	Balance
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9) THIXOTROPIC LIQUID AUTOMATIC DISHWASHING COMPOSITION

5	C ₁₂ -C ₁₄ fatty acid	0	-	0.5%
	Block co-polymer surfactant	1.5	-	15.0%
	Sodium citrate	0	-	12%
	Sodium tripolyphosphate	0	-	15%
	Sodium carbonate	0	-	8%
10	Aluminium tristearate	0	-	0.1%
	Sodium cumene sulphonate	0	-	1.7%
	Polyacrylate thickener	1.32	-	2.5%
	Sodium polyacrylate	2.4	-	6.0%
	Boric acid	0	-	4.0%
15	Sodium formate	0	-	0.45%
	Calcium formate	0	-	0.2%
	Sodium n-decydiphenyl oxide disulphonate	0	-	4.0%
	Monoethanol amine (MEA)	0	-	1.86%
20	Sodium hydroxide (50%)	1.9	-	9.3%
	1,2-Propanediol	0	-	9.4%
	Enzymes	0.0001	-	0.1%
	Suds suppressor, dye, perfumes, water	Balance		

25 10) LIQUID AUTOMATIC DISHWASHING COMPOSITION

	Alcohol ethoxylate	0	-	20%
	Fatty acid ester sulphonate	0	-	30%
	Sodium dodecyl sulphate	0	-	20%
	Alkyl polyglycoside	0	-	21%
30	Oleic acid	0	-	10%
	Sodium disilicate monohydrate	18	-	33%
	Sodium citrate dihydrate	18	-	33%

	Sodium stearate	0	-	2.5%
	Sodium perborate monohydrate	0	-	13%
	Tetraacetylenethylenediamine (TAED)	0	-	8%
	Maleic acid/acrylic acid copolymer	4	-	8%
5	Enzymes	0.0001	-	0.1%

11) LIQUID AUTOMATIC DISHWASHING COMPOSITION CONTAINING PROTECTED BLEACH PARTICLES

	Sodium silicate	5	-	10%
	Tetrapotassium pyrophosphate	15	-	25%
10	Sodium triphosphate	0	-	2%
	Potassium carbonate	0	-	2%
	Protected bleach particles, e.g. chlorine	5	-	10%
	Polymeric thickener	0.7	-	1.5%
15	Potassium hydroxide	0	-	2%
	Enzymes	0.0001	-	0.1%
	Water	Balance		

11) Automatic dishwashing compositions as described in 1), 2), 3), 4), 6) and 10), wherein perborate is replaced by per-
20 carbonate.

12) Automatic dishwashing compositions as described in 1) - 6) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching",
25 Nature 369, 1994, pp. 637-639.

The glucose oxidase of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the glucose oxidase may be added
30 in an amount corresponding to 0.00001-1 mg (calculated as pure enzyme protein) of glucose oxidase per liter of wash/dishwash liquor.

The present invention is further illustrated in the following examples which are not intended to be in any way limiting to the scope of the invention as claimed.

EXAMPLE 1

5 Glucose Oxidase from Cladosporium oxysporum.

The glucose oxidase of the invention may be produced by the method described in this example.

Fermentation:

The glucose oxidase producing strain, e.g., Clado-
10 sporium oxysporum, CBS 163.94, was inoculated on agar plates containing a medium of the following composition:

	Yeast extract, Difco	4 g/l
	K ₂ HPO ₄	1 g/l
	MgSO ₄	0.5 g/l
15	Glucose	15 g/l
	Distilled water	1000 ml
	Agar, Merck	15 g/l
	pH adjustment to 7.2.	

Growth at 26°C for 9 days.

20 The agar was cut into pieces and transferred aseptically to shake flasks, each flask containing 100 ml of inoculation medium of the following composition:

	Cornsteep liquor	24 g/l
	Glucose	24 g/l
25	Water ad 1000 ml, pH adjustment to pH 5.5.	
	soybean oil	0.5 ml/100 ml medium
	CaCO ₃	0.5 g/100 ml medium

The flasks were incubated on a rotary shaker at 250 rpm, at 26°C, for 24 hours. The contents of these flasks were
30 used for the inoculation of the production flasks which contained the following production medium (100 ml per flask):

34

	NaNO ₃	3 g/l
	KH ₂ PO ₄	1 g/l
	MgSO ₄ , 7H ₂ O	0.5 g/l
	KCl	0.5 g/l
5	FeSO ₄ , 7H ₂ O, 1%	1 ml/l
	Sucrose	30 g/l
	Yeast extract	5 g/l
	Distilled water	1000 ml
	Pluronic	0.1 ml/l
10	pH adjustment to pH 6.4.	

Growth at 26°C, 60 hours, 250 rpm.

Using the above described method a culture of Cladosporium oxysporum, CBS 163.94, gave a fermentation result of 5 UNITS/ml.

15 Purification and Characterization:

After separation of the solid material by centrifugation the glucose oxidase was filtrated and concentrated using a Filtron™ ultrafiltration module with a 10 kDa cut off membrane and filtrated again. The enzyme solution was then
20 purified by hydrophobic interaction using the following conditions:

	Temperature:	25°C
	pH:	5.0 (adjusted with H ₃ PO ₄)
	Addition of salt:	32.5% (w/w) (NH ₄) ₂ SO ₄
25	Stirring:	30 min.
	Centrifugation:	Heraeus Sepatech Varifuge 20 RS 12000 x g; 30 min.; 25°C (Precipitation discarded)
	Resin:	Phenyl Sepharose CL-4B equilibrated with 30 30% (w/w) (NH ₄) ₂ SO ₄
	Addition of resin:	1.0 g (dry sucked)/5.0 g supernatant
	Stirring:	30 min followed by dry suction
	Wash of resin:	30% (w/w) (NH ₄) ₂ SO ₄ 6-10 ml/g resin
	Stirring:	15 min. followed by dry suction
35	Elution:	Piperazine 20 mM pH 5.0 (HCl) 3-5 ml/g

resin
Stirring: 30 min. followed by dry suction
Elution: Repeated as described above.

After hydrophobic interaction purification the
5 glucose oxidase solution was ultrafiltrated again, this time
using a 20 kDa cut off membrane. Finally a ionexchange chroma-
tography purification took place using a Pharmacia LKB
HPLC/25°C system using piperazine as buffer.

Yield: a supernatant of 2850 ml gave a final solution
10 (after purification) of 30 ml, corresponding to approximately
53 mg glucose oxidase protein (A_{200}).

The MW and the pI of the glucose oxidase were
estimated to 90-100 kDa (SDS-PAGE) and 4.1-4.3 (IEF) respect-
ively. The found molecular weight (MW) may be that of a subunit
15 (monomer).

pH Profile: The pH profile was performed using a glucose
oxidase preparation which after fermentation as described above
was ultrafiltrated using a 10 kDa cut off membrane and then
precipitated with ethanol 55% (w/w) whereafter it was ultrafil-
20 trated again resulting in a preparation with an activity of 200
UNITS/ml. The preparation was used to determine the pH profile
previously described in this specification.

EXAMPLE 2

Glucose Oxidase from *C. oxysporum* for Gluten Strengthening

25 The dynamic shear storage modulus G' and the visco-
elastic phase angle δ (defined as previously described) were
measured in the gluten from 4 doughs, which were treated with
different conditioners. The conditioners were added to the
flour before dough mixing. The gluten was washed out of the
30 flour dough containing the conditioner after the flour dough
had been incubated at 32°C for 1½ hours. The results of the
tests are presented in Figs. 2 and 3. The following symbols

were used:

□: a dough with no added conditioner (= reference);

▽: a dough containing 20 ppm KBrO_3 . KBrO_3 is a traditional dough conditioner;

5 ◇: a dough containing 500 UNITS per kg of flour of a glucose oxidase from A. niger;

△: a dough containing 500 UNITS per kg of flour of a glucose oxidase of the invention, obtained as described in Example 1;

It can be seen from Fig. 2 that surprisingly the
10 storage modulus G' of the gluten treated with the glucose oxidase of the invention is significantly higher than the G' of the gluten treated with the traditional glucose oxidase, dosed with the same amount of units, and also higher than the G' of the traditional dough conditioner KBrO_3 . This result suggests
15 that the gluten, thereby the dough, is significantly strengthened by addition of the glucose oxidase of the invention.

Fig. 3 shows that the traditional dough conditioner KBrO_3 as expected lowers the phase angle δ compared to the
20 reference with no added conditioner; this indicates a more elastic rheological property. This decrease in phase angle is also found with the traditional glucose oxidase from A. niger. However, when using the glucose oxidase of the invention the phase angle δ is decreased even more when compared with the
25 traditional conditioners.

The above demonstrated effects of the glucose oxidase of the invention can also be found when additionally glucose is added to the dough.

EXAMPLE 3

30 Baking Trial

Recipe (wheat flour pan bread):

Wheat flour	100 %
Yeast	2.5 %

Salt 2 %
 Sugar 1 %
 Water 54.5 %

Flour type: Flour without ascorbic acid (Intermill, Schweiz).

5 Preparation of bread, procedure:

1. Dough mixing (Spiral mixer)

3 min. at 700 RPM

6 min. at 1400 RPM

the mixing time was determined and adjusted by a skilled baker
 10 so as to obtain an optimum dough consistence under the testing
 conditions used.

2. Fermentation 28°C - 60% RH, 40 min.

3. Sheeting, moulding and panning.

4. Fermentation 32°C - 80% RH, 70 min.

15 5. Baking: 230°C, 35 min.

Evaluation of Dough and Baked Bread

Dough and baked breads described in this example were
 evaluated as follows:

Loaf specific volume: The mean value of 4 loaves volume were
 20 measured using the traditional rape seed method. The specific
 volume was calculated as volume ml per g bread. The specific
 volume of the control (without enzyme) was defined as 100. The
 relative specific volume index was calculated as:

$$\begin{array}{l}
 \text{specific vol. of 4 loaves} \\
 25 \quad \text{Specific vol. index} = \frac{\text{-----}}{\text{spec. vol. of 4 control loaves}} * 100
 \end{array}$$

The dough stickiness, firmness, extensibility,
 elasticity and flavour as well as the colour/crust crispy/crumb
 structure of the breads were evaluated according to the follow-
 30 ing score system:

5	<u>Dough Stickiness:</u>	almost liquid	1
		too sticky	2
		sticky	3
		normal	4
		dry	5
10	<u>Dough Firmness:</u>	very soft	1
		too soft	2
		soft/good	3
		normal	4
		firm	5
		too firm	6
15	<u>Dough Extensibility:</u>	very short	1
		short	2
		normal	3
		good	4
		long	5
		too long	6
20	<u>Dough Elasticity:</u>	very strong	1
		strong	2
		normal	3
		good	4
		weak	5
		too weak	6
25	<u>Dough Flavour:</u>	less	2
		normal	3
		good	4
30	<u>Bread Colour:</u>	very light	1
		light	2
		normal	3
		good	4

	very good	5
	too dark	6
<u>Crust Crispy:</u>	too rubbery	1
	rubbery	2
5	normal	3
	good/crispy	4
	very good	5
	too crispy	6
<u>Crumb Structure:</u>	very poor	1
10	poor	2
	non-uniform	3
	uniform/good	4
	very good	5

Shock test: After the second fermentation the breads were
 15 exposed to an over fermentation of 10 min., 32°C, 80% RH, (i.e.
 for these breads the second fermentation lasted 80 min. in
 all), whereafter a pan containing the dough was dropped from a
 height of 20 cm. The dough was then baked (225°C, 30 min.) and
 the volume of the resulting bread was determined.

20 Table 1
Evaluation Doughs

	Glucose Oxidase UNITS/kg flour	0	250	500
	Stickiness	4	4.5	5
	Firmness	4	4.5	5
25	Extensibility	3	2.5	2
	Elasticity	3	3	3
	Flavour	3	3	3

It can be seen from Table 1 that glucose oxidase
 showed good effects on dough consistency such as decreased
 30 stickiness and improved firmness thereby strengthening the

gluten net and thereby improving the stability.

Table 2

Evaluation Breads.

5	Glucose Oxidase UNITS/kg flour	0	250	500
	Specific volume index %	100	110	120
	Colour	3	4	5
	Crust crispy	3	4	5
	Crumb structure	3	4	2.5
10	After shock test Specific volume index %	100	117	136

It can be seen from Table 2 that glucose oxidase of the invention has a significant effect on improving the baking quality of flour. The specific volume increased between 10% to 15 20%, and the breads also performed very well after shock test. The colour, the crust crispy and the crumb structure were also improved after adding glucose oxidase, only the crumb structure did not perform so satisfactory if the glucose oxidase is dosed higher than 400-500 UNITS per kg flour.

International Application N : PCT/

/

MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 3, line 10 of the description ***A. IDENTIFICATION OF DEPOSIT ***Further deposits are identified on an additional sheet ☐ *

Name of depositary institution *

CENTRAALBUREAU VOOR SCHIMMELCULTURES

Address of depositary institution (including postal code and country) *

Oosterstraat 1, Postbus 273, NL-3740 AG Barn, Nether-
land

Date of deposit *

25 March 1994

Accession Number *

CBS 163.94

B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC / Regulation 3.25 of Australia Statutory Rules 1991 No 71).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (If the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS *** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)
(Authorized Officer)☐ The date of receipt (from the applicant) by the International Bureau is

was

(Authorized Officer)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: NOVO NORDISK A/S
- 5 (B) STREET: Novo Alle
- (C) CITY: Bagsvaerd
- (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): DK-2880
- (G) TELEPHONE: +45 44 44 88 88
- 10 (H) TELEFAX: +45 44 49 32 56
- (I) TELEX: 37304

(ii) TITLE OF INVENTION: ALKALINE GLUCOSE OXIDASE

(iii) NUMBER OF SEQUENCES: 1

15 (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

20 (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- 25 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Ser Pro Ala Glu Pro Pro Val Val Ala Ala

1

5

10

CLAIMS

1. A glucose oxidase **characterized** by:
 - (a) A pH-optimum in the range pH 6-7, having more than 75% of maximum activity at pH 8, determined at 30°C with D-glucose as substrate.
2. A glucose oxidase according to claim 1, **characterized** by displaying immunochemical cross-reactivity with an antibody raised against a glucose oxidase obtainable from a strain of the genus Cladosporium.
- 10 3. A glucose oxidase according to claim 2, wherein said glucose oxidase is obtainable from a strain of Cladosporium oxysporum.
4. A glucose oxidase according to either of claims 2-3, wherein said glucose oxidase is obtainable from the strain
15 Cladosporium oxysporum, CBS 163.94.
5. A glucose oxidase according to any of claims 1-4, comprising the following amino acid sequence in the N-terminal: Ala-Ser-Pro-Ala-Glu-Pro-Pro-Val-Val-Ala-Ala (SEQ ID No. 1) or a glucose oxidase being at least 70% homologous with the
20 amino acid sequence (SEQ ID No. 1) in the N-terminal.
6. A process for the production of glucose oxidase, **characterized** by comprising cultivation in a suitable nutrient medium of a glucose oxidase producing strain of Cladosporium oxysporum, preferably CBS 163.94.
- 25 7. A process according to claim 6, wherein said cultivation is performed at a temperature in the range 20-30°C.
8. A bleaching composition comprising a glucose oxidase according to claims 1-5 and a substrate for the glucose

oxidase.

9. A bleaching composition according to claim 8, which additionally comprises a peroxidase.

10. A bleaching composition according to claim 9, which additionally comprises an oxidizable substrate such as an organic compound, such as a phenolic compound, such as p-hydroxybenzenesulfonic acid.

11. A detergent composition comprising a surfactant, a glucose oxidase according to any of claims 1-5 and a substrate for the glucose oxidase.

12. A detergent composition according to claim 11, which additionally comprises a peroxidase.

13. A detergent composition according to claim 12, which additionally comprises an oxidizable substrate such as an organic compound, such as a phenolic compound, such as p-hydroxybenzenesulfonic acid.

14. A dough conditioner or bread-improving composition comprising a glucose oxidase according to any of claims 1-5.

15. A pre-mix for baked products which comprises a glucose oxidase according to any of claims 1-5.

16. Use of a glucose oxidase according to any of claims 1-5 for improving properties of a dough and/or a baked product made from a dough.

17. Use of a glucose oxidase according to any of claims 1-5 for improving properties of gluten.

18. Use of a glucose oxidase according to any of claims 1-5 for a personal care product.

19. A DNA construct comprising a DNA sequence encoding a glucose oxidase according to any one of claims 1-5.

5 20. A recombinant expression vector which carries a DNA construct according to claim 19.

21. A cell which is transformed with a DNA construct according to claim 18 or a vector according to claim 20.

22. A cell according to claim 21, which is a microor-
10 ganism.

23. A cell according to claim 22, which is a bacterium or a fungus.

24. A method of producing a glucose oxidase according to any one of claims 1-5, wherein a cell according to any one
15 of claims 21-23 is cultured under conditions conducive to the production of the glucose oxidase and the glucose oxidase is subsequently recovered from the culture.

AMENDED CLAIMS

[received by the International Bureau on 26 September 1995 (26.09.95);
original claim 5 cancelled; remaining claims unchanged (1 page)]

1. A glucose oxidase **characterized** by:

(a) A pH-optimum in the range pH 6-7, having more than 75% of maximum activity at pH 8, determined at 30°C with D-glucose as substrate.

2. A glucose oxidase according to claim 1, **characterized** by displaying immunochemical cross-reactivity with an antibody raised against a glucose oxidase obtainable from a strain of the genus Cladosporium.

10 3. A glucose oxidase according to claim 2, wherein said glucose oxidase is obtainable from a strain of Cladosporium oxysporum.

4. A glucose oxidase according to either of claims 2-3, wherein said glucose oxidase is obtainable from the strain
15 Cladosporium oxysporum, CBS 163.94.

~~5. A glucose oxidase according to any of claims 1-4, comprising the following amino acid sequence in the N-terminal: Ala-Ser-Pro-Ala-Glu-Pro-Pro-Val-Val-Ala-Ala (SEQ ID No. 1) or a glucose oxidase being at least 70% homologous with the
20 amino acid sequence (SEQ ID No. 1) in the N-terminal.~~

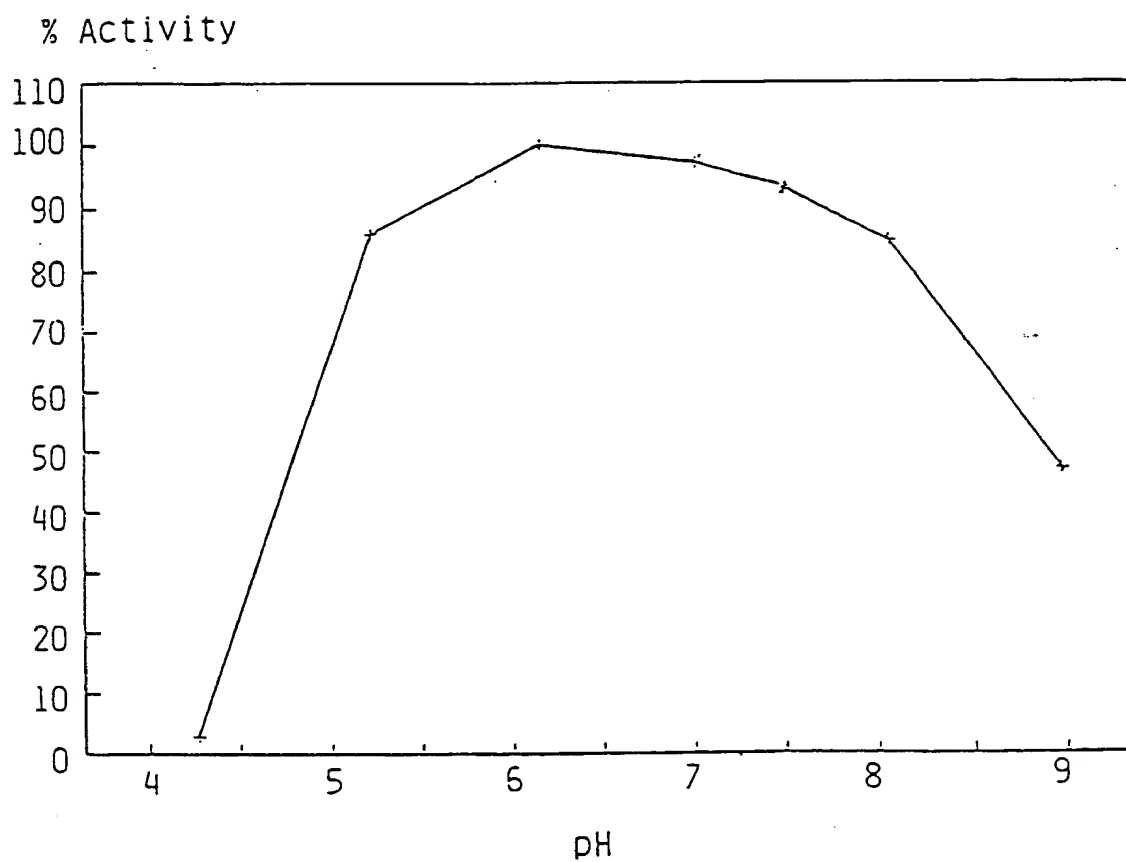
6. A process for the production of glucose oxidase, **characterized** by comprising cultivation in a suitable nutrient medium of a glucose oxidase producing strain of Cladosporium oxysporum, preferably CBS 163.94.

25 7. A process according to claim 6, wherein said cultivation is performed at a temperature in the range 20-30°C.

8. A bleaching composition comprising a glucose oxidase according to claims 1-5 and a substrate for the glucose

STATEMENT UNDER ARTICLE 19

Claim 5 in the present application was cancelled because the shown amino acid sequence in said claim and in the description (p. 9, 10, 42, 43) was in error.

1/3**Fig. 1**

2/3

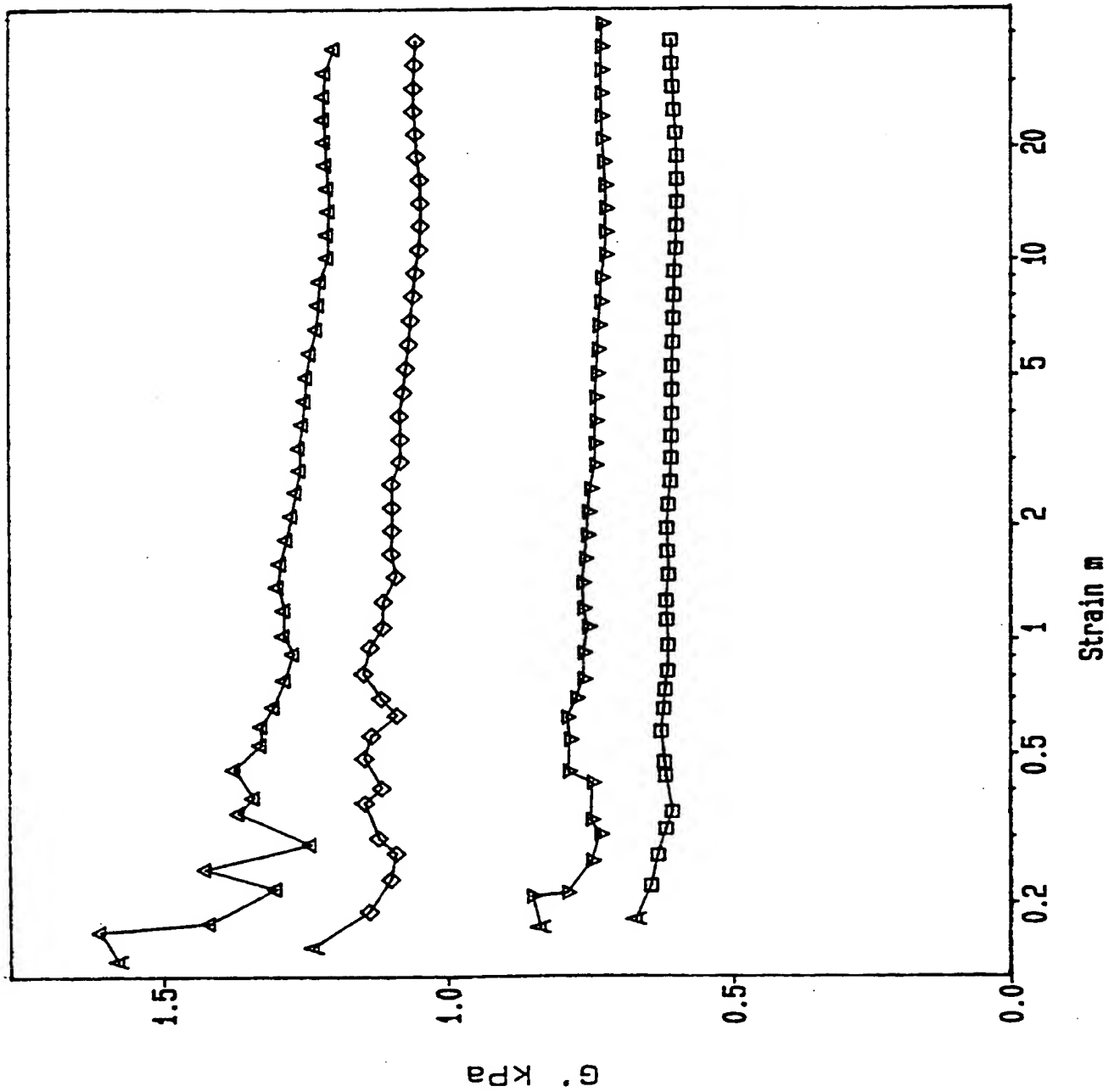


Fig. 2

3/3

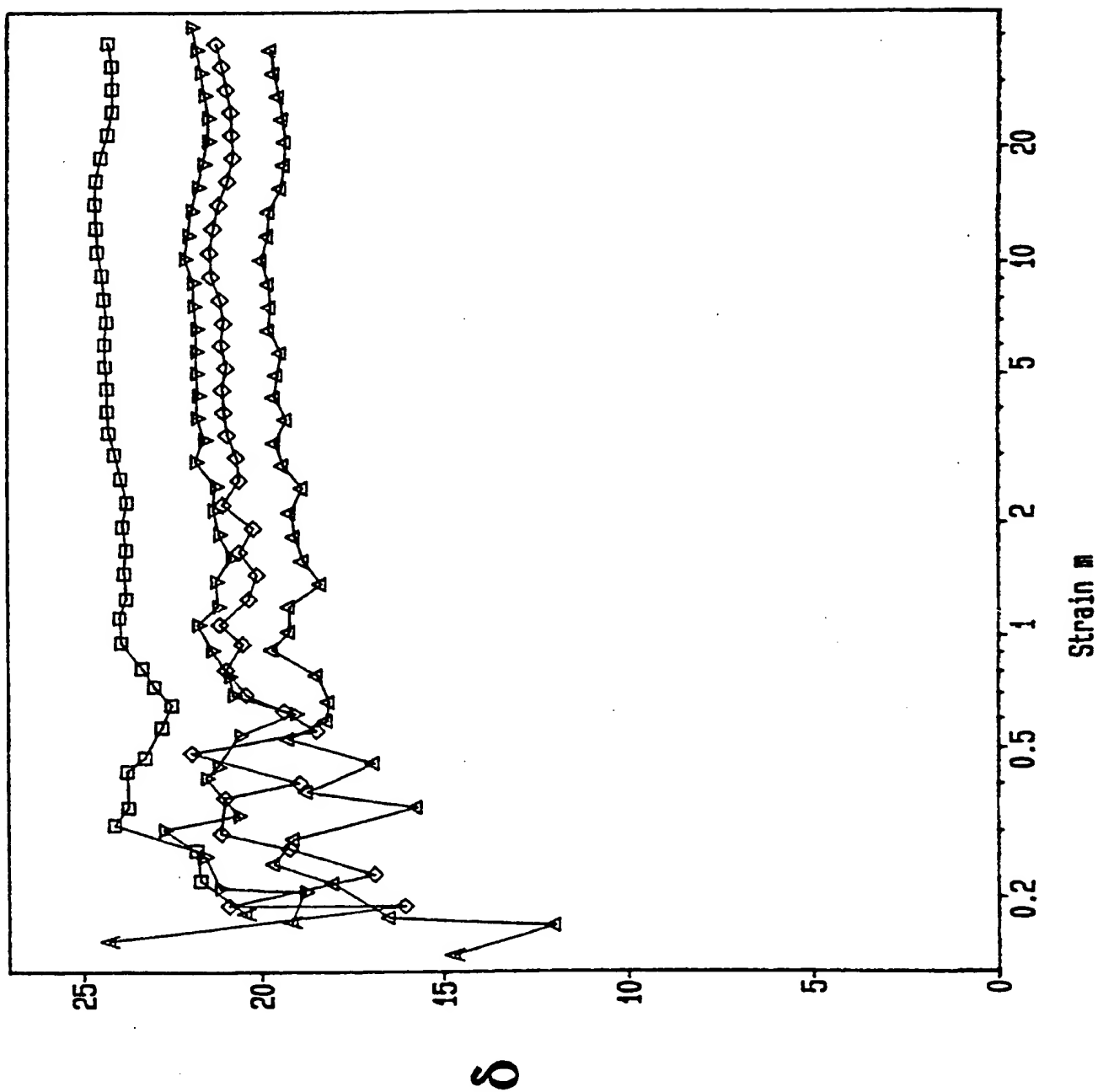


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00178

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/04, C12N 15/53, C11D 3/386, A21D 2/26
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C11D, A21D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, DBA, WPI, EDOC, STN FILE REG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Service, File 351, World Patent Index 89-94, Dialog accession no. 009937307, WPI accession no. 94-205019/25, OJI PAPER CO: "Alkali-resistant pyranose oxidase prodn. - comprises culturing Coriolus genus esp. Coriolus hirsutus IFO-4917 strain" JP 6141854, A, 940524, 9425 (Basic)	1-7, 18-24
Y	--	8-17
Y	US 5288746 A (K. PRAMOD), 22 February 1994 (22.02.94), column 9, line 36 - line 41	8-13
A	--	1-7

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

16 August 1995

Date of mailing of the international search report

23 -08- 1995

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 95/00178

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0338452 A1 (CULTOR LTD.), 25 October 1989 (25.10.89)	14-17
A	<p style="text-align: center;">-- -----</p>	1-7

INTERNATIONAL SEARCH REPORT

31/07/95

International application No.

PCT/DK 95/00178

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US-A-	5288746	22/02/94	EP-A-	0603931	29/06/94
			JP-A-	6234997	23/08/94
EP-A1-	0338452	25/10/89	SE-T3-	0338452	
			CA-A-	1335635	23/05/95
			DE-U-	6890285	22/10/92
			JP-A-	1312956	18/12/89
			NO-B-	177212	02/05/95
			US-A-	4990343	05/02/91